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ABSTRACT

Embryonic Stem (ES) cells can give rise, *in vivo*, to the ectodermal, endodermal and mesodermal germ layers and, *in vitro*, can differentiate into multiple cell lineages, offering broad perspectives in regenerative medicine. Understanding the molecular mechanisms governing ES cell commitment is an essential challenge in this field. The Mitogen Activated Protein Kinase (MAPK) pathways, ERK, JNK and p38MAPK, are able to regulate ES commitment from early steps of the process to mature differentiated cells. Whereas the ERK pathway inhibits the self-renewal of ES cells, upon commitment this pathway is involved in the development of extraembryonic tissues, in early mesoderm differentiation and in the formation of mature adipocytes; p38MAPK displays a large spectrum of action from neurons to adipocytes and JNK is involved in both ectoderm and primitive endoderm differentiations. Furthermore, for a given pathway, several of these effects are isoform-dependent, revealing the complexity of the cellular response to activation of MAPK pathways. Regarding tissue regeneration, the potential outcome of systematic analysis of the function of different MAPKs in different ES cell differentiation programs is discussed.

Introduction

ES cells can give rise, *in vivo*, to the ectodermal, endodermal and mesodermal germ layers and, *in vitro*, can differentiate into multiple cell lineages, offering broad perspectives in regenerative medicine **(**see for extensive reviews on mouse and human ES cells [1] and [2]). One existing limitation to the therapeutical use of these cells is that, *in vitro*, the capacity to orientate ES cells in a given lineage is often limited to a small proportion of cells. In fact, the cellular population obtained is usually a mixture of different specialized cells. Thus, understanding the molecular mechanisms governing commitment of ES cell to specific lineages is an essential challenge in this field.

Mouse ES cells can be maintained, *in vitro*, in an undifferentiated state in the presence of a cytokine, the leukaemia inhibitory factor (LIF) [3, 4]. Mouse ES cell self-renewal is dependent on: i) intracellular pathways initiated by LIF and by either serum or Bone Morphogenetic Protein 2 or 4 (BMP2 or 4), ii) activity of transcription factors involved in the development of the embryo: Oct4, Nanog, Sox2 and FoxD3 [5]. Removing LIF and adding appropriate differentiation reagents result in the commitment of ES cells into a variety of mature differentiated cell types (see [6] and [1] for reviews). Straight-forward synthesis of the literature regarding ES cell differentiation is hindered by the widely-varied deployment of differentiation protocols (embryoid bodies, attached ES cells, etc ...) that lack or include fetal bovine serum and the use of specific inducers such as retinoic acid (RA). While neural differentiation of ES cells is apparently a default program [7], this differentiation program is markedly enhanced by incubation with RA and/or differentiation in serum-free media [8] [9, 10]. By contrast, ES cell differentiation in the presence of fetal bovine serum, but the absence of RA, yields efficient differentiation to cardiomyocytes with few neurons [9, 11]. It is almost certain that different differentiation protocols and inducers will selectively activate distinct signalling pathways that activate cell lineage-specific genetic programs to bring about the

observed enrichment in differentiated cell populations. Yet, the precise molecular identity of these signalling pathways controlling cell differentiation of ES cells remains poorly understood. The purpose of this article is to review the recent studies that identify the role of Mitogen Activated Protein Kinases (MAPKs) in the *in vitro* differentiation of ES cells.

The MAP Kinase signal transduction pathways

Cells respond to extracellular signals by engaging a variety of intracellular signalling pathways, which trigger both immediate and long-term cell responses. The latter activate cascades that signal to the nucleus and regulate gene expression. The signalling pathways leading to activation of mitogen-activated protein kinases (MAPKs) and their downstream effects on gene regulation represent a paradigm in cellular signalling (see reviews [12, 13]). The MAPK family comprise four groups of proteins: extracellular signal-regulated kinases (ERKs) 1 and 2; ERK5; c-Jun amino-terminal kinases (JNKs) 1, 2 and 3; and p38MAPK α, β, γ, and δ; where each isoform is encoded by its own gene. Much of the present understandings of the MAPKs, especially regarding ES cell signalling, arise from the study of ERK1/2, JNK and p38MAPK proteins. These protein serine/threonine kinases are regulated by phosphorylation cascades organized in specific modules comprised of two additional protein kinases activated in series and leading to activation of a specific MAP Kinase: a MAP Kinase Kinase (MAPKK), which phosphorylates a specific MAPK, and a MAP Kinase Kinase Kinase (MAPKKK), which phosphorylates a specific MAPKK (**Figure 1**). Besides the activities of the different components of the cascades themselves, there are two others important means to specifically regulate these signalling pathways: interfering with the scaffolding proteins or MAPK phosphatases that are specific for each pathway.

Taking advantage of the development of specific chemical inhibitors for each MAPK pathway, numerous investigations have explored their biological functions and demonstrated

their involvement in a wide variety of cellular functions. These multiple functions are dependent on the pathway that is activated and on the cellular model analysed. In addition, the duration of the stimulus can also affect the cellular response. A wide panel of different stimuli are able to activate the MAPK pathways, but a good correlation has been found between the types of stimulus and the function assigned to the pathway. Schematically, ERK is preferentially activated by mitogens such as the serum or growth factors and, accordingly, this pathway is an important regulator of cell cycle and cell proliferation; whereas p38MAPK and JNK are responsive to various stress stimuli from UV to cytokines, and constitute important mediators of cellular responses to these stimuli (see for extensive reviews [14] and [12]; and more recently: [15], [16] and [17]). For example, the JNK pathway is the mediator of apoptosis induced by TNF-α. However, this growth factor is also able to activate the NFκ-B pathway, which, in turn, inhibits JNK. Therefore the cellular response will result from the combinatorial action of distinct signalling pathways.

Regarding the process of differentiation, the role of MAPKs is extremely complex and depends on multiple parameters. The complexity is due, firstly, to the biological process itself, which, in general, involves distinct, successive steps. Furthermore, each of these steps can be modulated by MAPKs leading, sometimes, to opposite effects. Probably because of this complexity, most of the tools used for these studies have found their limitations. With regard to small molecule inhibitors of protein kinases, inhibitors of a given pathway differ widely in their inhibitory potency and specificity [18]. Therefore, interfering with a given pathway with chemical inhibitors can induce different biological effects by virtue of simple non-specific effects. Alternatively, investigators have constructed cell lines from various cellular models, overexpressing dominant-negative or activated forms of the genes encoding the components of MAPK pathways. While often informative, these experiments are not totally conclusive because of the complexity of the differentiation process and the possible cross talk between

the different pathways in such conditions. Finally, homozygous knockouts of several components of MAPK pathways are now available, both *in vivo*, in mice, and *in vitro*, in ES cell lines [19]. Whereas targeted gene disruption in animals may unveil important biological functions, they also have limits, especially when the knock-out is lethal during early embryogenesis. Recently, the study of ES cells bearing disrupted MAPK genes revealed that: no role could been assigned to these pathways in undifferentiated ES cells as MAPK pathways are apparently dispensable for ES cell self-renewal and cell cycle (see below). By contrast, new biological functions can be attributed to these proteins in the modulation of ES cell lineage commitment, which is the subject of the subsequent sections of this review.

The ERK pathway

Engagement of the gp130 cytokine receptor subunit by the LIF generates two intracellular signalling pathways: on one hand, the JAK-STAT3 pathway, which is required for ES self-renewal, and, on the other hand, the ERK pathway. Surprisingly and in contrast to most cultured cell lines, undifferentiated ES cells do not require the ERK pathway for normal cell cycle, proliferation and self-renewal [20-22]. In fact, inhibition of this pathway actually promotes self-renewal of murine ES cells. These observations are in agreement with the study of K-Ras$^{-/-}$ ES cells, the absence of this gene leading to downregulation of the ERK pathway. These cells display LIF-independent capacity to grow undifferentiated [23]. Therefore, it appears that the self-renewal signal downstream of LIF is a finely tuned balance of positive (via STAT3 proteins) and negative (via the ERK pathway) effectors [24].

The dominant role of the ERK pathway becomes apparent upon differentiation, both *in vivo* and *in vitro*. Interfering with the ERK signalling pathway, for example by knock-out of the upstream activator Grb2, leads to inhibition of primitive endoderm [25, 26] and trophectoderm formations [27]. These *in vivo* observations have also been reproduced in

cultures of the corresponding deficient ES cells. Surprisingly, activation of the ERK pathway by an oncogenic Ras also interferes with extraembryonic endoderm differentiation of embryonic stem cells [28]. It is likely that a critical level of activation of the ERK pathway is necessary for this early commitment and that up or down variations of this threshold level have deleterious consequences. Beside these early functions in commitment, not much was known about the role of ERK in ES cell differentiation. Recently, our laboratory showed that the treatment of ES cells by retinoic acid, which is required for induction of neurogenesis and adipogenesis, activates the ERK pathway. Inhibition of ERK activation, using specific chemical inhibitors during this period, results in a strong inhibition of adipocyte formation without affecting neurogenesis [29]. Furthermore, by studying knock-out animals and fibroblasts, the role of ERK in adipogenesis appears to be limited to ERK1, with no role for ERK2 in this process [30]. To gain genetic evidence of the role of ERK1 in adipocyte differentiation of ES cells and to analyse its function in other cell lineages, it would be of interest to generate and test the differentiation capacities of $ERK1^{-/-}$ ES cells.

Interestingly, while $ERK1^{-/-}$ mice are viable and fertile [31], disruption of ERK2 is embryonic lethal due to defective placenta formation, trophectoderm and mesoderm differentiations [32, 33]. These findings confirm the direct role of the ERK pathway early during the embryonic development. Furthermore, they demonstrateERK1 and ERK2 have distinct biological functions. While, as expected, ERK2 disruption does not interfere with proliferation of undifferentiated ES cells, no apparent mesoderm-derived lineages can be observed upon ES commitment (B. Binetruy and F. Bost, personal observation), suggesting that ERK2 is necessary at an early step of ES cell commitment. Since $ERK^{-/-}$mice present normal mesoderm differentiation -except for adipocyte formation-, it is likely that the defect of $ERK2^{-/-}$ ES cells in mesoderm commitment takes place earlier than the defective adipogenesis of $ERK1^{-/-}$ cells.

Many defined ERK substrates are transcriptional regulators (reviewed in [34]), but what specific molecular mechanisms are controlled by each of the ERK isoforms during mesoderm formation remains to be determined. Regarding adipocyte differentiation, the positive role of the ERK pathway is blurred by works demonstrating that the adipocyte-specific transcription factor PPARγ is a substrate of ERK and that this phosphorylation decreases its transcriptional activity and inhibits adipocyte differentiation [35, 36]. Owing to the known role of ERK in cell proliferation, one could reconcile these contradictory results by hypothesizing that the function of ERK in adipogenesis is dictated by the window of time during which it is activated. Early in the program, ERK activity is increased for a proliferative step, while later, it must be inactivated to prevent PPARγ phosphorylation. This model is supported by the fact that the expression of MKP-1, the phosphatase that inhibits ERK, is augmented in mature adipocytes [37].

The p38MAPK pathway

Among the four p38MAPK isoforms, α, β, γ, and δ, only the knock-out of p38α is embryonic lethal [38], the others presenting no apparent phenotype. The lethality is due to both embryonic defect and a lack of erythropoietin expression. It has been shown that the p38MAPK pathway plays a crucial role during early mammalian somite development and myotome formation, at E9.5 of the embryo development, by signaling to the MEF2 transcriptional regulators [39]. The p38α isoform is the only one expressed in ES cells [40]. Whereas no role has been assigned to p38MAPK in undifferentiated ES cells, p38MAPK activation is involved in the early apoptosis observed in a fraction of ES cells early on upon induction of differentiation [41]. Although p38MAPK protein expression is constant, two waves of p38MAPK activity characterise the ES cell differentiation process, one between day 2 to 5 [11, 41] and one, latter on, between day 12 to 16 [42]. By analysing both the effects of

p38MAPK-specific pharmaceutical inhibitors and the *in vitro* differentiation capacities of ES cells deficient for the p38α gene, our laboratory found that these activities regulate ES cell commitment. The early peak of P38MAPK activity controls a switch between cardiomyogenesis (p38MAPK activity turned on) and neurogenesis (p38MAPK off) [11], while the second one inhibits adipogenesis [42].

Interestingly, RA treatment inhibited both the first peak of p38MAPK activation and the *in vitro* formation of cardiomyocytes. Therefore, it is likely that RA blocks cardiomyogenesis in ES cells via p38MAPK inhibition. Few studies have shown that RA modulates MAPK activity, however, a recent report demonstrated that RA inhibits cyclic stretch induced activity in neonatal cardiomyocytes via MAPK inhibition [43]. This inhibition could be due to an increase of expression of the MAPK phosphatases MKP-1 and -2 by RA. Either deletion of p38MAPK or specific inhibition of its peak of activity partially mimicked the *in vitro* RA inhibition of cardiomyogenesis and reduced expression of cardiomyocyte markers, including the important transcription factorMEF2C [11], which acts on many genes encoding cardiac structural proteins. Interestingly, p38MAPK is a well-known regulator of MEF2C [44-47], suggesting that the p38MAPK effect could be directly due to MEF2C regulation. Consistent with this hypothesis, a role for p38α in various aspects of cardiomyogenesis including the regulation of cardiomyocyte differentiation, apoptosis, and hypertrophy has been described [48-50], and, accordingly, $p38\alpha^{-/-}$ embryos present a massive reduction of the myocardiac muscle attributed to a defect in placental development [51].

In PC12 and P19 cell lines, p38MAPK activation is required for neurite formation and neuron survival during late stages of differentiation [52, 53]. In fact, the role of p38MAPK in these cells is restricted to the late stages of differentiation. Indeed, PC12 cells are already committed into the neuronal lineage [54] and P19 is a multipotent embryonic cell line [55, 56] that terminally differentiates into neurons after RA treatment. By contrast, analysis of the role

of p38MAPK in the early stages of neuron differentiation, during ES cells commitment, revealed an opposite function for this kinase. Inhibition of p38MAPK using specific inhibitors or p38$\alpha^{-/-}$ cells is sufficient to induce, spontaneously, a high level of neurogenesis [11].

Altogether these results suggest that p38MAPK may exert different roles depending on the stage of neuronal differentiation: inhibitory during cell commitment and anti-apoptotic during the late stages of differentiation. It is very likely that the molecular mechanisms underlying these distinct functions are different and their identification should be of a great interest for the development of ES cells in therapeutic applications.

The JNK pathway

Whereas single knock-out of individual JNK genes have no effect on mice, *jnk*1$^{-/-}$*jnk*2$^{-/-}$ mice undergo mid-gestational embryonic lethality associated with defects in neural tube closure and deregulated neural apoptosis [57, 58]. The deletion of JNK1, but not JNK2, leads to the resistance to high-fat diet induced obesity and the absence of JNK1 leads to a better insulin sensitivity attributed to a reduced phosphorylation of IRS-1 on Ser307 [59]. However, if the JNK pathway is involved in insulin signalling, there is no evidence for a role of this pathway in adipocyte differentiation. Similar to the ERK and p38 MAPK pathways, the JNK family of MAPKs are not required for self-renewal or maintenance of ES cells [8]. A variety of approaches have defined a role for the JNK pathway in differentiation of ES cells as well as P19 embryonal carcinoma cells to neural and extraembryonic endoderm lineages. We recently employed ES cells derived from mice bearing disrupted *jnk*1, *jnk*2 or *jnk*3 to define a requirement for JNK1 in retinoic acid-induced neurogenesis [8]. Importantly, the lack of neurogenesis by JNK1$^{-/-}$ ES cells was associated with enhanced induction of an epithelial differentiation program evidenced by increased E-cadherin. In addition, the expression of Wnt-4, Wnt-6 and BMP4 were markedly increased in the JNK1$^{-/-}$ cultures, consistent with a

role for specific Wnts and BMP4 as members of a key lineage commitment switch in ES cell differentiation [60] [61] [7]. A role for the JNKs in neural differentiation of ES cells is consistent with the observation of similarly reduced neural differentiation in ES cells deficient for the JNK pathway scaffold protein, JSAP [62]. Thus, these studies support a model where JNK1 activity represses a Wnt-4/Wnt-6 and BMP4 signaling axis that would otherwise direct the cells towards an epithelial lineage.

The earliest two extraembryonic cell lineages are the trophectoderm and the primitive endoderm, which will form the placenta and yolk sac, respectively. Following implantation of early mammalian embryos, primitive endoderm differentiates to visceral endoderm and parietal endoderm; these tissues reside on the periphery of embryoid bodies formed *in vitro* by ES cells and embryonal carcinoma cells. Several groups have used P19 cells to unveil the requirement of a JNK signalling pathway in the retinoic acid-stimulated differentiation of these cells to primitive endoderm lineages [63]. In addition, our own recent studies reveal that retinoic acid-stimulated expression of a variety of visceral and parietal endoderm lineage markers (GATA4, GATA6, Sox17, disabled 2, α-fetoprotein) are inhibited in $JNK1^{-/-}$ and $JNK2^{-/-}$ ES cell-derived embryoid bodies (L. Heasley, unpublished observation). In the P19 cell model system, signaling by the heterotrimeric G protein, G_{13}, and stimulation of the activity of p115RhoGEF is required for primitive endoderm differentiation [64]. Moreover, a recent study by Kashef et al [65] demonstrated that the G_{13}-interacting JNK pathway scaffold protein, JLP, is markedly induced by retinoic acid in P19 cells. Thus, a key regulatory step in retinoic acid-stimulated primitive endoderm differentiation appears to be the increased expression of a specific scaffold protein to assemble a G_{13}-stimulated JNK module.

Conclusions and perspective

MAPK pathways are able to regulate both the early embryonic development and the ES cell commitment from early steps of the process to mature differentiated cells (the various effects are summarised in **Figure 2**). The ERK pathway is mainly involved in mesoderm differentiation, especially in adipogenesis, with both positive and negative effects. p38MAPK displays a large spectrum of action from neurons to adipocytes and JNK is involved in both ectoderm and primitive endoderm differentiations. Furthermore, for a given pathway, these effects are isoform-dependent, revealing the complexity of the cellular response to activation of these pathways. Notably, several lineages tested are affected by more than one transduction pathway. Adipogenesis is controlled by both ERK and p38MAPK and neurogenesis is controlled by both p38MAPK and JNK. These observations are reminiscent of our recent studies in PC12 pheochromocytoma cells, indicating that complex differentiation programs such as neurogenesis will involve the integration of multiple signal pathways [66]. Even the induction of a neural-specific gene such as neurofilament light chain in these cells involves the concerted action of the ERK and JNK signal pathways [66]. Thus, it is equally likely that distinct ES cell lineage commitment programs will be regulated through the integrated action of two or more MAPK families. Cross talks between MAPK pathways can be either synergistic -ERK/JNK in neural differentiation of PC12- or antagonistic -p38MAPK/ERK in adipocyte differentiation of ES cells. Yet, taking advantage of the availability of the various MAPKs-disrupted ES cell lines, the role of MAPKs in differentiation of numerous other cell types needs to be investigated.

Altough the precise molecular mechanisms underlying the various MAPK functions in ES cell commitment are unknown, they must eventually lead to activation of cell lineages-specific genetic programs. Recently, important features of regulation of gene expression in ES cells have been unveiled. In undifferentiated mouse and human ES cells, the transcriptional repressors Polycomb Group proteins (PcG) repress numerous developmental regulators that,

once de-repressed, are able to trigger ES cells to undergo differentiation [67-69]. The repression is due to the PcG-induced hypermethylation of histone proteins in nucleosomes occupying key-regions of the gene promoters. Upon induction of differentiation this process is reverted and developmental regulators are expressed. These results underline the critical role of chromatin modification in ES cell commitment. Interestingly, MAPKs induce a dynamic change in histone phosphoacetylation during ES cell differentiation [70]. Yet, we do not know whether this regulation is related to the biological role of MAPKs in ES cell commitment.

Dominant signal pathways that control ES cell lineage commitment *in vitro* do not always translate to critical roles for these MAPK pathways unveiled with gene knockout approaches during mouse development. As an example, JNK1-deficient ES cells fail to undergo neurogenesis [8]. Yet, the JNK1 knockout mouse develops normally. Also, our own studies (L. Heasley, unpublished observations) reveal inhibited extraembryonic endoderm differentiation by ES cells deficient for either JNK1 or JNK2. Yet, marked perturbations in extraembryonic endoderm fate specification is not observed, even in embryos lacking both JNK1 and JNK2. The implication from these discrepancies is that studies of signal transduction in ES cells will not always provide information that directly transfers to mouse development. One potential outcome of systematic analysis of the function of different MAPKs in different ES cell differentiation programs is that inhibitors to specific protein kinases may be used to "trap" key cellular intermediates in ES cell lineage commitment pathways. These intermediate cell types may serve as more useful reagents for tissue regeneration *in vivo* than the fully differentiated cell types. Obviously, a progenitor may be more likely to reconstitute multiple cell types within a tissue relative to a more differentiated cell type. Since the ultimate goal of tissue regeneration is the use of *in vitro* committed stem cells, it is crucial to understand and control the various steps of their differentiation. The pharmacological regulation of MAP Kinase activities could participate in the obtaining of

such cells. Furthermore, this strategy could be extended to other protein kinase families. Finally, whereas this review focuses on mouse ES cells, the role of signal transduction pathways and of MAP Kinases, in particular, in human ES cell differentiation is still largely unexplored. Interestingly, and by contrast to mouse ES cells, a recent work indicates that ERK could be necessary for the maintenance of human ES cell pluripotency [71].

In conclusion, despite the fact that not all ES cell findings may directly translate to dominant mechanisms of cell fate specification during development, they provide a highly valuable foundation of knowledge as tissue propagation from ES cells emerges as a discipline distinct from developmental biology.

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Legends to figures

Figure 1

Schematic representation of the main MAP Kinase signal transduction pathways and their regulators.

Figure 2

Proposed model for the *in vivo* and *in vitro* MAPK effects on development (in italics) and ES cell differentiation. This model is deducted from the literature (see text).

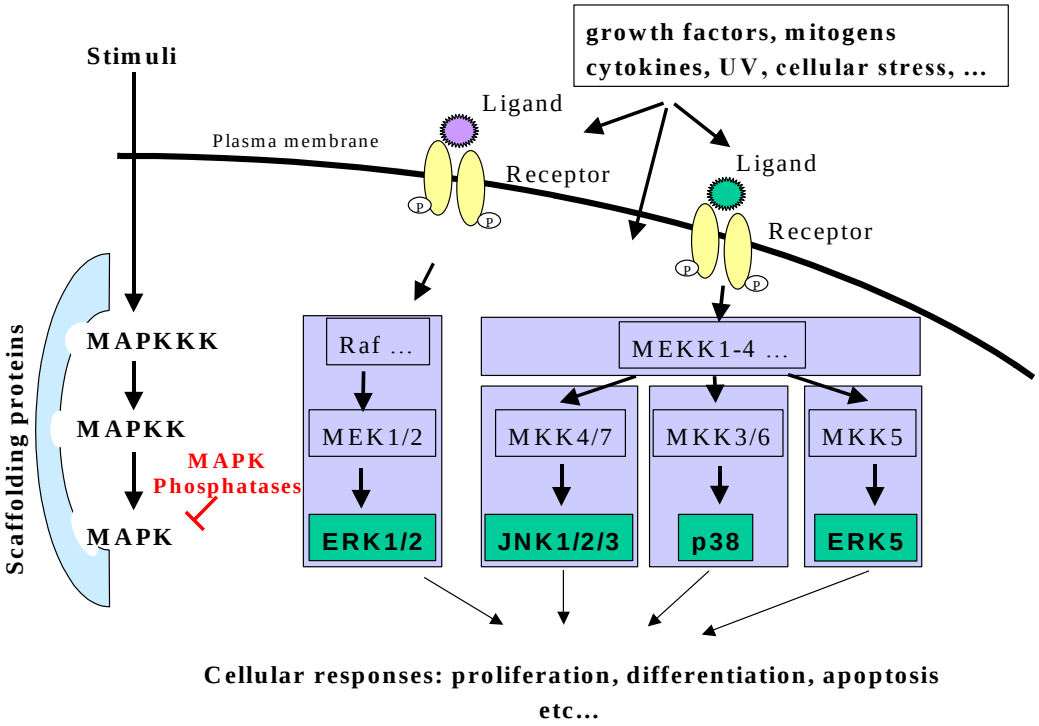
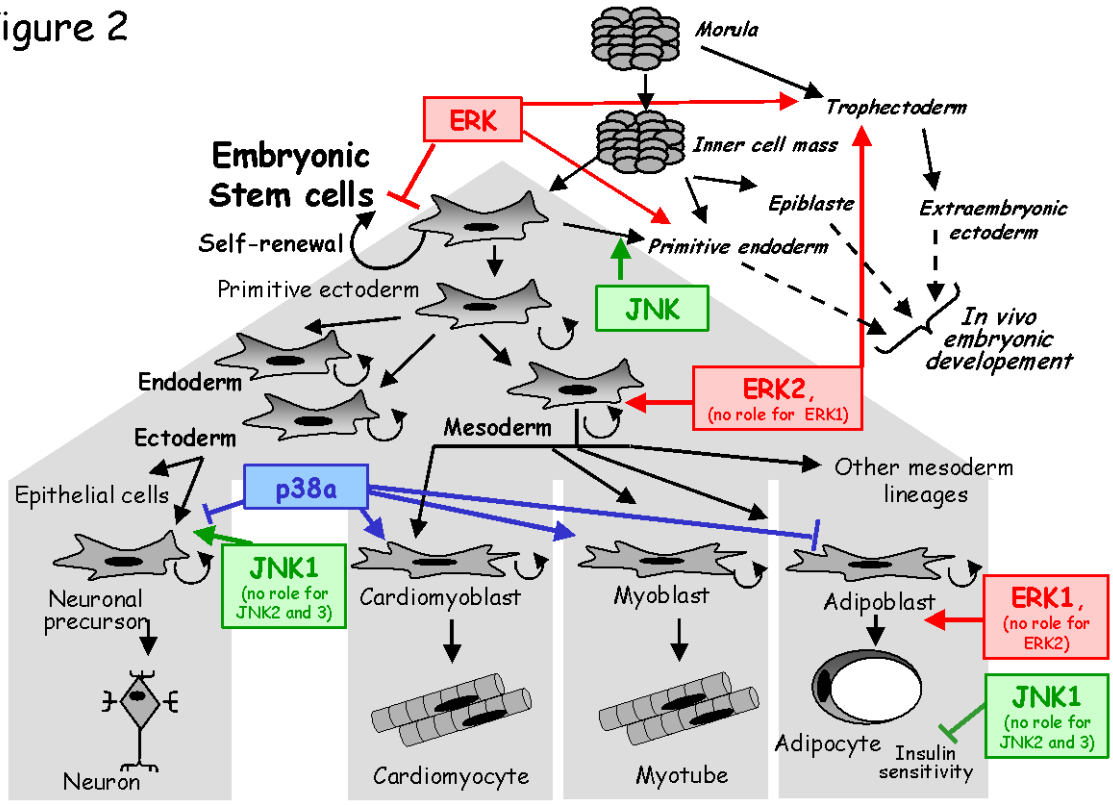

growth factors, mitogens
cytokines, UV, cellular stress, ...
Stimuli
Plasma membrane
Ligand
Receptor
Ligand
Receptor
P
P
P
P
Scaffolding proteins
MAPKKK
MAPKK
MAPK
MAPK
Phosphatases
Raf ...
MEK1/2
ERK1/2
MEKK1-4 ...
MKK4/7
JNK1/2/3
MKK3/6
p38
MKK5
ERK5
Cellular responses: proliferation, differentiation, apoptosis
etc...

Figure 1

Figure 2


Morula
Trophectoderm
ERK
Inner cell mass
Embryonic Stem cells
Epiblaste
Extraembryonic ectoderm
Self-renewal
Primitive endoderm
Primitive ectoderm
JNK
In vivo embryonic developement
Endoderm
ERK2, (no role for ERK1)
Ectoderm
Mesoderm
Other mesoderm lineages
Epithelial cells
p38a
JNK1 (no role for JNK2 and 3)
Neuronal precurson
Cardiomyoblast
Myoblast
Adipoblast
ERK1, (no role for ERK2)
JNK1 (no role for JNK2 and 3)
Neuron
Cardiomyocyte
Myotube
Adipocyte
Insulin sensitivity